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ORIGINAL ARTICLE

A multilocus study of pine grosbeak phylogeography supports the pattern of greater intercontinental divergence in Holarctic boreal forest birds than in birds inhabiting other high-latitude habitats

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ABSTRACT

Aim Boreal forest bird species appear to be divided into lineages endemic to each northern continent, in contrast to Holarctic species living in open habitats. For example, the three-toed woodpecker (*Picoides tridactylus*) and the winter wren (*Troglodytes troglodytes*) have divergent Nearctic and Palaearctic mitochondrial DNA clades. Furthermore, in these species, the next closest relative of the Nearctic/Palaearctic sister lineages is the Nearctic clade, suggesting that the Palaearctic may have been colonized from the Nearctic. The aim of this study is to test this pattern of intercontinental divergence and colonization in another Holarctic boreal forest resident – the pine grosbeak (*Pinicola enucleator*).

Location The Holarctic.

Methods We sequenced the mitochondrial ND2 gene and *Z*-specific intron 9 of the ACO1 gene for 74 pine grosbeaks collected across the Holarctic. The sequences were used to reconstruct the phylogeographical history of this species using maximum likelihood analysis.

Results We discovered two distinct mitochondrial and *Z*-specific lineages in the Nearctic and one in the Palaearctic. The two Nearctic mtDNA lineages, one in the northern boreal forest and one in south-western mountain forest, were more closely related to each other than either was to the Palaearctic clade. Two Nearctic Z-chromosome clades were sympatric in the boreal and south-western mountain forests. Unlike the topology of the mtDNA tree, the relationship among the Z-chromosome clades was the same as in the three-toed woodpecker and winter wren [Nearctic (Nearctic, Palaearctic)]. The Palaearctic Z-chromosome clade had much lower genetic diversity and a single-peak mismatch distribution with a mean < 25% of that for either Nearctic region, both of which had ragged mismatch distributions.

Main conclusions Our data suggest that, similar to the other boreal forest species, the pine grosbeak has divergent lineages in each northern continent and could have colonized the Palaearctic from the Nearctic. Compared with many Holarctic birds inhabiting open habitats, boreal forest species appear to be more differentiated, possibly because the boreal forests of the Nearctic and Palaearctic have been isolated since the Pliocene (3.5 Ma).

Keywords
Boreal forest birds, Holarctic, mtDNA, phylogeography, pine grosbeak, *Pinicola enucleator*, Z-chromosome.

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INTRODUCTION

The two northern continents share over 100 avian species (Peters *et al.*, 2008). The majority of high-latitude Holarctic species are associated with wetlands, or other open, non-forested habitats (Table 1). Recent studies reveal either little or no genetic differentiation between Nearctic and Palaearctic populations in several such species – the common raven (*Corvus corax*; Omland *et al.*, 2000), peregrine falcon (*Falco peregrinus*; Berlin & Ellegren, 2001), herring gull (*Larus argentatus*; Liebers *et al.*, 2004), mallard (*Anas platyrhynchos*; Kulikova *et al.*, 2005), gyrfalcon (*Falco rusticolus*; Johnson *et al.*, 2007), gadwall (*Anas strepera*; Peters *et al.*, 2008), bank swallow (*Riparia riparia*; Pavlova *et al.*, 2008), horned lark (*Eremophila alpestris*), willow ptarmigan (*Lagopus lagopus*) and rock ptarmigan (*Lagopus muta*; S.V. Drovetski *et al.*, unpublished data) (for scientific nomenclature see Dickinson, 2003). In contrast to open-country species, the number of Holarctic species associated with boreal forests is remarkably low considering the vast expanse of this habitat on both continents and the number of Holarctic genera (Table 1). We may hypothesize that this is the result of a much longer separation of Asian and American boreal forests compared with that of open habitats. The boreal forests were separated by the opening of the Bering Strait in the Late Pliocene, *c.* 3.5 Ma, whereas treeless habitats were connected as recently as a few thousand years ago during the last glaciation (Sanmartín *et al.*, 2001). Until recently, 10 Holarctic boreal forest species were recognized: the northern goshawk (*Accipiter gentilis*), great grey owl (*Strix nebulosa*), northern hawk owl (*Surnia ulula*), boreal owl (*Aegolius funereus*), three-toed woodpecker (*Picoides tridactylus*), winter wren (*Troglodytes troglodytes*), Bohemian waxwing (*Bombycilla garrulus*), red crossbill (*Loxia curvirostra*), white-winged crossbill (*Loxia leucoptera*) and pine grosbeak (*Pinicola enucleator*).

Zink *et al.* (2002) showed that Nearctic and Palaearctic three-toed woodpeckers were reciprocally monophyletic and differed by 3.8% sequence divergence across several mitochondrial DNA (mtDNA) genes, and, consequently, the American three-toed woodpecker was recognized as a distinct species (*Picoides dorsalis*; Banks *et al.*, 2003). Of the three lineages of woodpeckers with three toes (two species of three-toed woodpeckers and the black-backed woodpecker, *Picoides arcticus*), two are Nearctic endemics. The American three-toed woodpecker has twice the nucleotide diversity of the Palaearctic representatives.

Drovetski *et al.* (2004a) showed that the winter wren has at least six divergent mtDNA lineages, four in the Palaearctic and two in the Nearctic. The divergence at ND2 among these clades varied between 3.0 and 8.8%. Two of the most divergent lineages in this complex were endemic to the Nearctic, and all Palaearctic lineages formed a derived clade sister to the widespread eastern and northern Nearctic clade. The divergence between each of the Palaearctic clades and their closest Nearctic clade was 5.6%. Therefore, similar to some non-avian boreal forest taxa (Lafontaine &

Table 1 Holarctic bird genera and species of high-latitude forest and non-forest habitats, demonstrating the difference in the species-to-genus ratio between the habitats. References provide genetic data that support or refute current taxonomy.

Holarctic genera	Holarctic species	Reference
Boreal forest taxa		
Falcipennis		Drovetski (2002)
Accipiter	*A. gentilis**	S.L. Talbot *et al.* (unpublished data)
Bubo		–
Strix	*S. nebularia*	–
Surnia	*S. ulula*	–
Aegolius	*A. funereus*	–
Dendrocopus/ *Picoides*		Benz *et al.* (2006)
Picoides		Zink *et al.* (2002)
Dryocopus		Benz *et al.* (2006)
Perisoreus		–
Nucifraga		–
Baeolophus/ *Lophophane*		Gill *et al.* (2005)
Poecile		Gill *et al.* (2005)
Certhia		Tietze *et al.* (2006)
Sitta		Zink *et al.* (2006a)
Troglodytes	*T. troglodytes**	Drovetski *et al.* (2004a)
Regulus		Päckert *et al.* (2003)
Turdus		Voelker *et al.* (2007)
Bombycilla	*B. garrulus**	–
Carpodacus		Arnaiz-Villena *et al.* (2001)
Loxia	*L. curvirostra**	Questiau *et al.* (1999)
	*L. leucoptera**	Parchman *et al.* (2007)
Pinicola	*P. enucleator**	This study
Carduelis		Arnaiz-Villena *et al.* (2007)
Coccothraustes		–
Non-forest high-latitude taxa		
Anser	*A. albifrons*	–
Anas	*A. platyrhynchos*	Kulikova *et al.* (2005)
	A. strepera	Peters *et al.* (2008)
	A. crecca	–
	A. acuta	–
	A. clypeata	–
Aythya	*A. marila*	–
Somateria	*S. molissima*	–
	S. spectabilis	–
Melanitta	*M. nigra*	–
	M. fusca	
Histrionicus	*H. histrionicus*	–
Clangula	*C. hyemalis*	–
Bucephala	*B. clangula*	–
Mergus	*M. merganser*	–
	M. serrator	–
Lagopus	*L. lagopus*	S.V. Drovetski *et al.* (unpublished data)

Table 1 Continued

Holarctic genera	Holarctic species	Reference
	L. muta	S.V. Drovetski *et al.* (unpublished data)
Gavia	*G. stellata*	–
	G. adamsii	–
Podiceps	*P. auritus*	–
	P. nigricollis	–
	P. grisigena	–
Pandion	*P. haliaetus*	–
Circus	*C. cyaneus*	–
Aquila	*A. chrysaetos*	–
Buteo	*B. lagopus*	Riesing *et al.* (2003)
Falco	*F. columbarius*	–
	F. perigrinus	Berlin & Ellegren (2001)
	F. rusticolus	Johnson *et al.* (2007)
	G. canadensis	–
Pluvialis	*P. squatarola*	–
Tringa		Pereira & Baker (2005)
Actitis		Pereira & Baker (2005)
Numenius	*N. phaeopus*	–
Arenaria	*A. interpres*	–
Calidris	*C. alpina**	Wenink *et al.* (1996)
Gallinago	*G. gallinago*/ *G. delicata**	S.V. Drovetski *et al.* (unpublished data)
Phalaropus	*Ph. lobatus*	Tavares & Baker (2008)
	Ph. fulicarius	
Larus	*L. argentatus*	Liebers *et al.* (2004)
	L. canus	–
Hydroprogne	*H. caspia*	–
Sterna	*S. hirundo*	–
	S. paradisaea	–
Chlidonias	*C. niger*	–
Stercorarius	*S. pomarinus*	–
S. parasiticus	*S. parasiticus*	–
S. longicaudus	*S. longicaudus*	–
Asio	*A. flammeus*	–
Bubo	*B. scandiacus*	Marthinsen *et al.* (2008)
Lanius	*L. excubitor*	–
Corvus	*C. corax*	Omland *et al.* (2000)
Eremophila	*E. alpestris*	S.V. Drovetski *et al.* (unpublished data)
Riparia	*R. riparia*	Pavlova *et al.* (2008)
Hirundo	*H. rustica*	Zink *et al.* (2006b)
Oenanthe	*O. oenanthe*	–
Calcarius	*C. lapponicus*	–
Plectrophenax	*P. nivalis*	–
Carduelis	*C. flammea*/ *C. hornemanni*	Seutin *et al.* (1995)

*Disagreements between the current taxonomy and available genetic data.

Wood, 1988), the three-toed woodpecker and winter wren have distinct sister lineages on each continent. Furthermore, they appear to have more deeply divergent lineages in the Nearctic and, thus, might have colonized the Palaearctic from the Nearctic. Differences in absolute amounts of genetic variation can be a flawed indicator of the direction of colonization because it is not easy to distinguish among selection, recent colonization and bottlenecks as the cause of the reduced variation. However, both species show a pattern of differentiation [Nearctic (Nearctic, Palaearctic)] that supports a hypothesis of Nearctic to Palaearctic colonization.

Limited data on the red crossbill (Questiau *et al.*, 1999), white-winged crossbill (Parchman *et al.*, 2007) and northern goshawk (S.L. Talbot, USGS Alaska Science Center, pers. comm.) also suggest the possibility of differentiation between the Palaearctic and Nearctic populations. Better sampling is required to assess the levels of intra- and intercontinental differentiation in these taxa.

The pine grosbeak is a Holarctic resident species of boreal-type forest. It exhibits limited seasonal movements and infrequent short-to-medium distance population irruptions (Adkisson, 1999). Although the pine grosbeak is abundant and widespread across both continents, the degree of phenotypic variation appears to be greater in the Nearctic than in the Palaearctic. The three Palaearctic subspecies are distinguished by their beak shape and size (Stepanyan, 2003). The beaks of Palaearctic pine grosbeaks are deeper and broader in birds of the eastern Palaearctic (*Pinicola enucleator kamtschatkensis*), especially those of Sakhalin Isand (*Pinicola enucleator sakhalinensis*), than in western Palaearctic birds (*Pinicola enucleator enucleator*).

Nearctic pine grosbeaks are divided into five subspecies that differ not only in their beak size and shape, but also in wing, tail and tarsus lengths and in overall body size (Adkisson, 1999). Furthermore, differences in vocalization suggest that the five Nearctic subspecies fall into two distinct groups. One group includes three southern subspecies inhabiting Queen Charlotte Island (*Pinicola enucleator carlottae*), the Rocky Mountains (*Pinicola enucleator montanus*) and the Sierra Nevada (*Pinicola enucleator californicus*). The second group consists of two northern subspecies: the widespread *Pinicola enucleator leucurus*, which breeds from Newfoundland to interior Alaska, and *Pinicola enucleator flammula*, which breeds along the Pacific coast from the Alaska Peninsula to British Columbia (Adkisson, 1999). This geographic pattern of phenotypic and vocal variation in the pine grosbeak suggests that this species may have a similar phylogeographic pattern to that identified in the three-toed woodpecker and the winter wren. Indeed, a recent study found that Queen Charlotte Island pine grosbeaks, which belong to the southern vocal group, were differentiated in their mtDNA cytochrome *b* sequences from Alaskan and Washington birds, which belong to the northern vocal group (Topp & Winker, 2008).

In this paper we use DNA sequences from the mitochondrial (mtDNA) ND2 gene (1041 bp) and intron 9 of the Z-chromosome-specific ACO1 gene (ACO1I9; 969 bp) to test for isolation of continental populations and a gene tree structure that is consistent with a Nearctic origin for the pine grosbeak.

MATERIALS AND METHODS

A total of 74 pine grosbeak tissue samples from various localities across the Holarctic were obtained from vouchered specimens deposited in museums (Fig. 1; Appendix S1 in Supporting Information). We also used 30 individuals of 23 finch species, which past work indicated would be likely outgroups (Drovetski *et al.*, 2009). Total genomic DNA extraction, the polymerase chain reaction (PCR) profile and primers for amplification of the complete mtDNA ND2 gene (1041 bp) followed methods described in Drovetski *et al.* (2004b). The ACO1I9 sequences were amplified with primers ACO1-I9F (CTGTGGGAATGCTGAGAGATTT) and ACO1-I9R (CTGCAGCAAGGCACAACAGT; Kimball *et al.*, 2009). We used the following touch-down PCR profile for amplification of ACO1I9: initial denaturation at 95 °C for 15 min, followed by five cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, extension at 72 °C for 45 s, followed by five cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 20 s, extension at 72 °C for 45 s, followed by five cycles of denaturation at 95 °C for 20 s, annealing at 54 °C for 20 s, extension at 72 °C for 45 s, followed by 20 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, extension at 72 °C for 45 s, followed by final extension at 72 °C for 10 min.

Polymerase chain reaction fragments were sequenced in both directions either at the DNA Analysis Facility at Yale University, equipped with an ABI 3730, 48-Capillary, Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA), or at the Swedish Museum of Natural History Molecular Systematics Laboratory using an ABI Prism 3100 automated DNA sequencer (Applied Biosystems Inc.). The sequences were aligned automatically in SEQUENCHER 4.2 (Gene Codes Corporation, Ann Arbor, MI USA). The alignment of ND2 sequences was unambiguous because there were no indels in these coding sequences. Alignment of ACO1I9 sequences required a minimal amount of editing because there were three short indels in pine grosbeak sequences (positions 136, 341, and 508–509), and a single-nucleotide indel (position 800) distinguished all pine grosbeak sequences from sequences of the Eurasian bullfinch (*Pyrrhula pyrrhula*), which was used as the closest available outgroup (Drovetski *et al.*, 2009).

In cases of heterozygous male ACO1I9 genotypes with multiple variable sites, alleles were sorted using DNASP 4.50.3 (Rozas *et al.*, 2003) and PHASE 2.1.1 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003) with the command line '-d1'. We verified all allele identifications manually. This was accomplished using known allele frequencies and linkage between nucleotide states at variable sites observed in hemizygous (female) or homozygous/single-variable-site (male) genotypes. We tried to minimize solutions that resulted in novel alleles not observed in the unambiguous part of our dataset. When multiple observed alleles could be extracted from an ambiguous genotype, we selected alleles with the greatest frequency among unambiguous alleles. All three methods produced identical results for all genotypes, including for 15 individuals with different-length alleles. When alleles differ in their length, nucleotide states in each allele can be identified directly by examining chromatograms. This examination showed that ambiguous sites were resolved correctly in all 15 individuals.

Maximum likelihood (ML), maximum parsimony (MP) and neighbour joining (NJ) analyses implemented in PAUP* 4.0 (Swofford, 1998) were used to reconstruct phylogenetic trees from nuclear and mitochondrial sequences. The best-fit model and its parameters for the final ML analysis were determined using the Akaike information criterion (AIC; Akaike, 1974) in

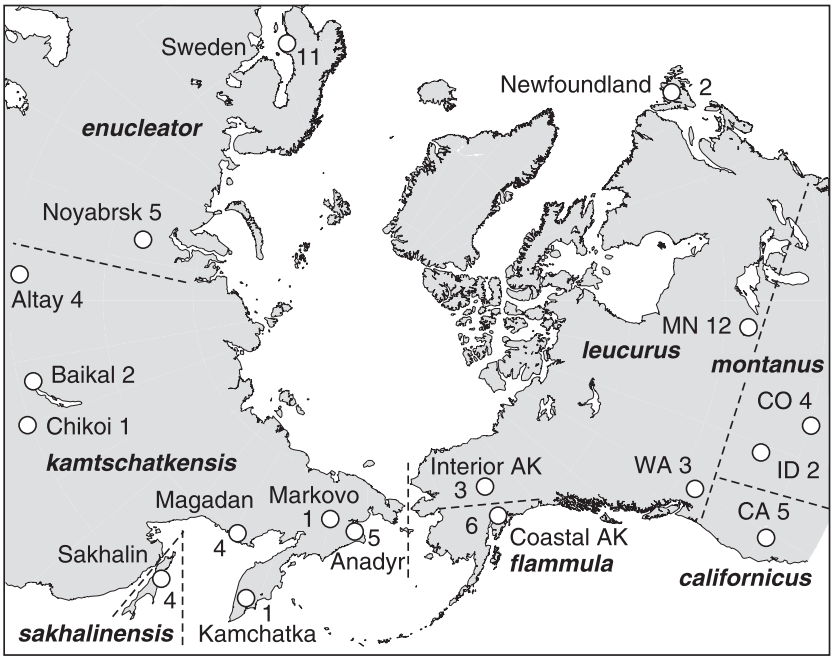

Figure 1 A polar stereographic (a conformal, azimuthal) projection map of sampling localities, sample sizes, and subspecies of pine grosbeak (*Pinicola enucleator*) in the Holarctic. AK, Alaska; MN, Minnesota; CO, Colorado; WA, Washington; ID, Idaho; CA, California.

Modeltest 3.7 (Posada & Crandall, 1998). Taxa were added randomly for both the full heuristic ML (10 replicates) and bootstrap (100 replicates) analyses. To determine if sequence evolution was clock-like, we compared likelihood scores of the ML tree with and without a molecular clock enforced; the likelihood ratio (LR) was computed as $-2(\ln L_{clock} - \ln L_{no\ clock})$ and was evaluated by assuming that the LR was chi-square distributed with the number of degrees of freedom (d.f.) equal to the number of taxa minus two (Nei & Kumar, 2000). A McDonald–Kreitman test (MK; McDonald & Kreitman, 1991) implemented in DnaSP 4.50.3 (Rozas *et al.*, 2003) was used to evaluate the influence of natural selection on sequence evolution. This software was also used to describe mismatch distributions, conduct additional tests, *R2* (Ramos-Onsins & Rozas, 2002) and Fu's F_S (Fu, 1997), and to estimate nucleotide (π_n) and haplotype (h) diversity, number of haplotypes and F_{ST}-values.

RESULTS

Analysis of ND2 sequences

The initial equally weighted MP and ML analyses, and the NJ analysis based on uncorrected '*p*' distances, which included 30 individuals of 22 outgroup species from the same family (Fringillidae; Appendix S1), identified three divergent, geographically concordant clades of pine grosbeak haplotypes (Appendix S2). The two sister clades of pine grosbeak were composed of Nearctic haplotypes from, respectively, the northern boreal forests (Alaska, Washington, Minnesota and Newfoundland) and south-western, mountain forests (California, Colorado, Idaho). The third clade included all of the Palaearctic haplotypes. To increase the precision of the substitution model estimate and thus improve the estimate of distances among haplotypes and clades, we constrained the Palaearctic haplotypes as a composite outgroup for the final ML analysis, which included only pine grosbeak haplotypes.

DnaSP identified 51 unique ND2 haplotypes among the 74 pine grosbeaks. The AIC selected the TIM+G model (Posada & Crandall, 1998) for the ND2 sequences. The TIM+G is a submodel of the general time-reversible (GTR) model (Tavaré, 1986), which weights A–T and C–G substitutions equally and includes the discrete-gamma model of substitution rates across sites (Yang, 1998). Guanine was under-represented and adenine and cytosine were over-represented in ND2 sequences of pine grosbeak (A = 31.31%, C = 34.20%, G = 9.72%, T = 24.77%; d.f. = 3, *G*-test $P < 0.0001$). All haplotypes shared this nucleotide bias, and there was no evidence of heterogeneity of base composition among them. NCBI accession numbers for our ND2 sequences are given in Appendix S1.

The ML analysis of pine grosbeak ND2 haplotypes resulted in a single tree that was not consistent with the molecular clock ($-\ln L_{no\text{-}clock} = 2238.24268$, $-\ln L_{clock} = 2278.92662$; $-2\Delta\ln L = 81.36788$, d.f. = 49, $P = 0.003$). This tree had the same topology (Fig. 2) as the pine grosbeak section of the tree produced by the initial analysis (Appendix S2). The branch length between the Palaearctic and northern Nearctic clades was 6.32%, and that between the Palaearctic and south-western Nearctic clades was 6.39%. The branch length between the two Nearctic clades was 2.21%. All three clades were supported by bootstrap values ≥ 80%. These data indicate a long-term isolation of Palaearctic and Nearctic pine grosbeaks, with more recent divergence between northern and south-western Nearctic clades. The MK test conducted using the Palaearctic and two Nearctic clades combined, as well as the test using the two Nearctic clades, revealed that ND2 sequence evolution in pine grosbeak is consistent with neutrality [neutrality index (NI) = 0.558, Fisher's exact $P = 0.28$ and NI = 1.742, $P = 0.70$, respectively]. Nucleotide diversity was 0.0049 ± 0.0006 for the northern Nearctic, 0.0027 ± 0.0006 for the south-western Nearctic, and 0.0036 ± 0.0005 for the Palaearctic (Table 2), and mismatch distribution means were 5.1, 2.8 and 3.7 respectively (Fig. 3a).

Within the south-western Nearctic clade, all haplotypes from California formed a recently derived clade. The branch length between this clade and its sister haplotype from the Rocky Mountains was only 0.1%, which represents a single mutation difference. Consequently, only about half of all bootstrap samples included this site, bringing the bootstrap value for the California clade to 49% (Fig. 2). Although the tree suggests a very recent divergence between California and Rocky Mountain pine grosbeaks, this result could be an artefact of our limited sampling in the south-western Nearctic.

Analysis of *Z*-specific ACO1I9 sequences

We were unable to amplify ACO1I9 for one individual (svd3109). Among the remaining 73 individuals (47 males and 26 females; total of 120 alleles), DnaSP identified 19 unique alleles. There was no evidence for recombination, as the nucleotide states at variable sites were in linkage disequilibrium. We used seven unique ACO1I9 alleles of the Eurasian bullfinch as a composite outgroup for the initial equally weighted MP and ML analyses, and the NJ analysis based on uncorrected '*p*' distances (Appendix S3). As in the case of ND2 data, this analysis identified one Palaearctic and two Nearctic clades. However, the two Nearctic clades were not sisters (one of them was the sister of the Palaearctic clade), and they were sympatric in both the northern boreal and south-western mountain parts of the species' range.

To estimate distances among clades and alleles more accurately and to test the sister relationship of the Palaearctic and one of the Nearctic clades, we used alleles from the most divergent Nearctic clade as a composite outgroup for the final phylogenetic analysis. The AIC selected the TrN+I model (Posada & Crandall, 1998) for the ACO1I9 sequences. The TrN+I is a submodel of the GTR model (Rodríguez *et al.*, 1990) in which transversions are weighted equally and some sites (85.5%) are invariable. Guanine and cytosine were under-represented and thymine was over-represented in ACO1I9 sequences of pine grosbeak (A = 25.29%, C = 17.96,

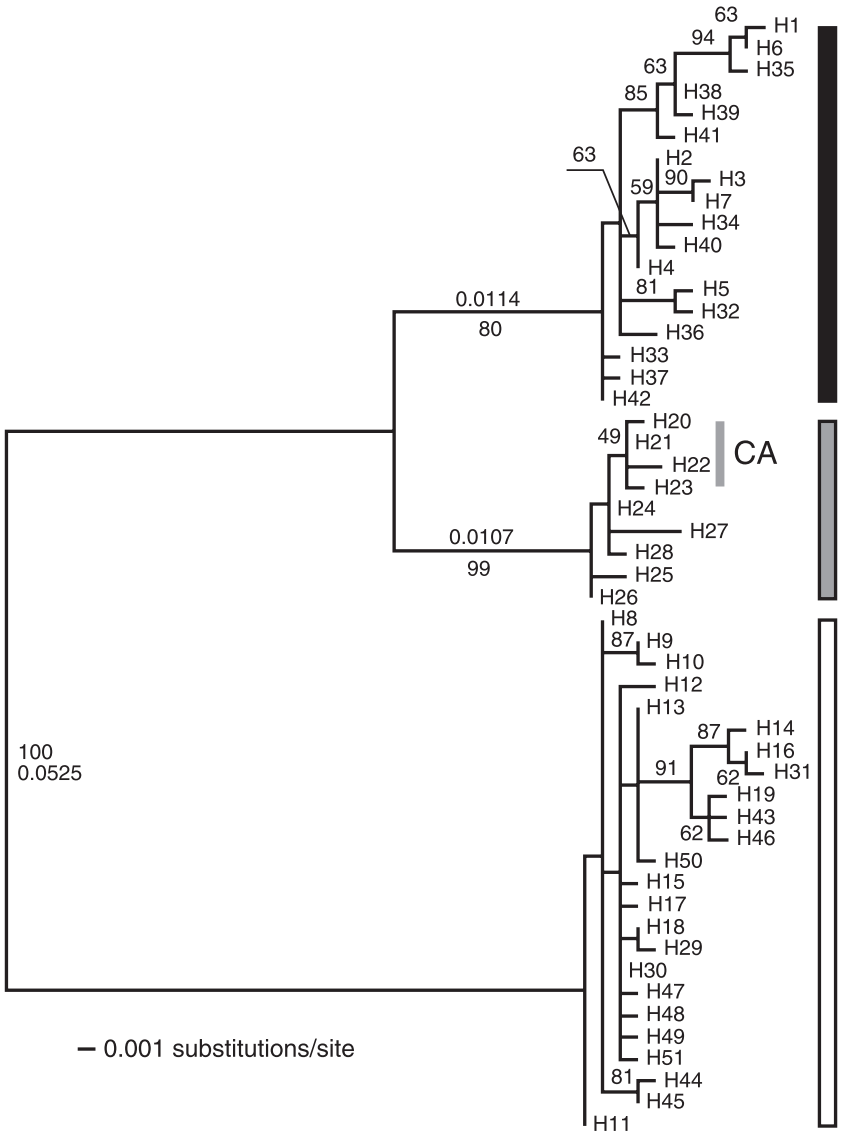

Figure 2 Maximum likelihood (ML) tree for ND2 haplotypes of pine grosbeak (*Pinicola enucleator*). Numbers next to branches identify bootstrap values (≥ 49) and branch lengths (< 1). Vertical bars next to haplotype names identify the geographic origin of the sequences: black, northern Nearctic; grey, south-western Nearctic; white, Palaearctic, CA, California.

G = 19.90, T = 36.85; d.f. = 3, *G*-test $P < 0.001$). All alleles shared this nucleotide bias, and there was no evidence of heterogeneity of base composition among them. NCBI accession numbers for our ACO1I9 sequences are given in Appendix S1.

The ML analysis of pine grosbeak ACO1I9 alleles resulted in a single tree (Fig. 4) that was consistent with the molecular clock ($-\ln L_{\text{no-clock}} = 1492.95162$, $-\ln L_{\text{clock}} = 1504.58272$; $-2\Delta\ln L = 23.2622$, d.f. = 17, $P = 0.14$). Palaearctic alleles formed a clade supported by a 67% bootstrap value. The sister relationship of the Palaearctic and one of the two Nearctic clades was supported by a 97% bootstrap value, and this Nearctic clade itself was supported by 64%. The branch length between the Palaearctic and its sister Nearctic clades was 0.22%, 29 times shorter than that between the Palaearctic and either of the Nearctic ND2 clades (Fig. 2). In contrast to the case for the ND2 tree, the two Nearctic ACO1I9 clades were not geographically concordant. The proportions of alleles from the northern and south-western parts of the species' range were similar in both Nearctic clades (20 northern: 6 south-western vs. 19:12; Fisher's exact $P = 0.26$), not permitting a geographical assignment of their origin. Pairwise F_{ST}-values for ACO1I9 sequences also provided no support for the differentiation of the northern and south-western regions that was observed in ND2 data. They were significant for comparisons of the Palaearctic with the northern ($F_{ST} = 0.491$, $P < 0.001$) and south-western ($F_{ST} = 0.597$, $P < 0.001$) Nearctic regions but were not significant for the comparison of the two Nearctic regions ($F_{ST} = 0.009$, $P = 0.252$).

Both Nearctic regions (northern and south-western) had an almost fourfold greater nucleotide diversity than the Palaearctic. This large difference in genetic variability was consistent across all localities (Table 2). Comparison of mismatch distributions showed that the Palaearctic had a distribution with a single peak and a mean pairwise difference of 1.35, whereas the northern and south-western Nearctic regions exhibited ragged distributions with much greater means (4.9

Table 2 ND2 variability of pine grosbeak (*Pinicola enucleator*) across localities and geographic regions of the Holarctic: number of haplotypes (n); haplotype diversity (h), nucleotide diversity (π_n) and their standard deviation (SD); Fu's F_S, *R2* and their *P*-values. 'nc' indicates insufficient sample size for computation.

Locality	n	$h \pm$ SD	$\pi_n \pm$ SD	Fu's F_S (P)	*R2* (P)
North Nearctic	25	0.943 ± 0.037	0.00491 ± 0.00058	−8.614 (0.001)	0.080 (0.049)
Alaska	9	0.917 ± 0.092	0.00560 ± 0.00131	−0.875 (0.253)	0.161 (0.373)
Minnesota	11	1.000 ± 0.039	0.00522 ± 0.00070	−6.620 (0.002)	0.089 (0.001)
Washington	3	nc	nc	nc	nc
Newfoundland	2	nc	nc	nc	nc
South-west Nearctic	11	0.964 ± 0.051	0.00269 ± 0.00058	−4.942 (0.001)	0.105 (0.022)
California	5	0.900 ± 0.161	0.00154 ± 0.00046	−1.405 (0.069)	0.187 (0.071)
Colorado	4	1.000 ± 0.177	0.00352 ± 0.00112	−0.946 (0.120)	0.242 (0.308)
Idaho	2	nc	nc	nc	nc
Palaearctic	38	0.945 ± 0.024	0.00358 ± 0.00046	−16.999 (0.000)	0.051 (0.007)
Altai	4	1.000 ± 0.177	0.00224 ± 0.00058	−1.622 (0.058)	0.208 (0.102)
Baikal/Chikoi	3	nc	nc	nc	nc
Markovo/Anadyr	6	1.000 ± 0.096	0.00467 ± 0.00104	−2.214 (0.051)	0.121 (0.015)
Kamchatka/Magadan	5	0.900 ± 0.161	0.00480 ± 0.00114	0.490 (0.510)	0.240 (0.520)
Noyabrsk	5	0.900 ± 0.161	0.00115 ± 0.00031	−1.938 (0.020)	0.163 (0.013)
Sakhalin	4	1.000 ± 0.177	0.00528 ± 0.00136	−0.399 (0.207)	0.227 (0.323)
Sweden	11	0.891 ± 0.074	0.00136 ± 0.00024	−4.054 (0.001)	0.093 (0.001)

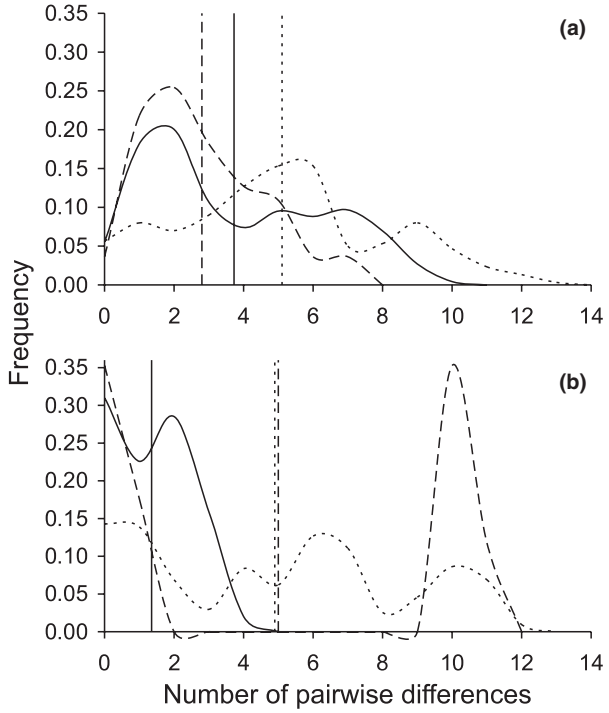

Figure 3 Mismatch distributions for (a) ND2 and (b) ACO1I9 sequences of pine grosbeak (*Pinicola enucleator*) for the Palaearctic (solid line), northern Nearctic (dotted line), and south-western Nearctic (dashed line). Vertical lines represent mean pairwise differences.

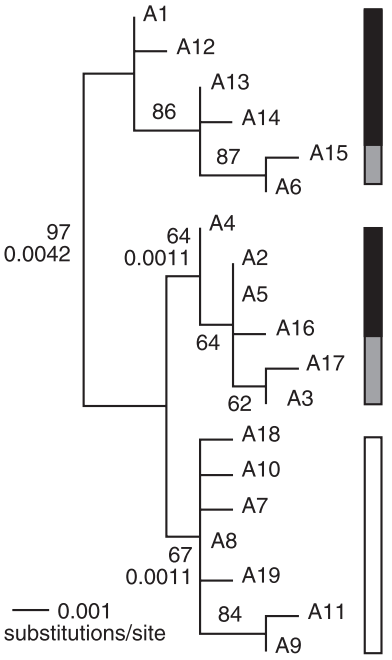

Figure 4 Maximum likelihood (ML) tree for ACO1I9 alleles of pine grosbeak (*Pinicola enucleator*). Numbers next to branches identify bootstrap values (≥ 62) and branch lengths (< 1). Vertical bars next to allele names identify the geographic origin of the sequences: black, northern Nearctic; grey, south-western Nearctic; white, Palaearctic.

and 5.0, respectively; Fig. 3b). The sympatry of the two divergent clades in both Nearctic regions was responsible for these large differences in genetic diversity between the Nearctic regions and the Palaearctic.

The differences in genetic diversity were less pronounced when the comparison was undertaken among clades rather than among geographic regions. The Palaearctic clade had a much higher nucleotide diversity (0.00140 ± 0.00012) than its sister Nearctic clade (0.00079 ± 0.00013) but a much lower

Table 3 ACO1I9 variability of pine grosbeak (*Pinicola enucleator*) across localities and geographic regions of the Holarctic: number of alleles (n_a); haplotype diversity (h), nucleotide diversity (π_n) and their standard deviation (SD); Fu's F_S, *R2* and their *P*-values. 'nc' indicates insufficient sample size for computation.

Locality	n_a	$h \pm$ SD	$\pi_n \pm$ SD	Fu's *Fs* (*P*)	*R2* (*P*)
North Nearctic	39	0.857 ± 0.028	0.00508 ± 0.00033	0.408 (0.59)	0.158 (0.92)
Alaska	14	0.890 ± 0.050	0.00485 ± 0.00091	0.033 (0.56)	0.190 (0.88)
Minnesota	17	0.875 ± 0.053	0.00517 ± 0.00058	0.236 (0.56)	0.168 (0.78)
Washington	4	0.833 ± 0.222	0.00569 ± 0.00270	1.655 (0.74)	0.382 (0.88)
Newfoundland	4	0.833 ± 0.222	0.00465 ± 0.00159	1.343 (0.66)	0.253 (0.40)
South-west Nearctic	18	0.647 ± 0.069	0.00518 ± 0.00083	7.277 (1.00)	0.227 (1.00)
California	9	0.556 ± 0.165	0.00426 ± 0.00168	3.796 (0.96)	0.179 (0.51)
Colorado	6	0.733 ± 0.155	0.00655 ± 0.00137	3.555 (0.94)	0.275 (0.89)
Idaho	3	nc	nc	nc	nc
Palaearctic	63	0.690 ± 0.042	0.00140 ± 0.00012	−0.678 (0.405)	0.096 (0.42)
Altai	7	0.667 ± 0.160	0.00108 ± 0.00041	0.110 (0.48)	0.246 (0.59)
Baikal/Chikoi	5	0.400 ± 0.237	0.00041 ± 0.00025	0.090 (0.19)	0.400 (0.72)
Markovo/Anadyr	10	0.733 ± 0.120	0.00131 ± 0.00033	−0.384 (0.31)	0.153 (0.13)
Kamchatka/ Magadan	8	0.821 ± 0.101	0.00199 ± 0.00038	0.081 (0.49)	0.241 (0.78)
Noyabrsk	8	0.464 ± 0.200	0.00052 ± 0.00024	−0.999 (0.08)	0.217 (0.38)
Sakhalin	6	0.533 ± 0.172	0.00110 ± 0.00036	1.723 (0.85)	0.267 (0.63)
Sweden	19	0.684 ± 0.070	0.00149 ± 0.00017	0.806 (0.70)	0.176 (0.78)

one than the other Nearctic clade (0.00236 ± 0.0017). None of the localities, geographical regions, or clades had significant *R2*- or Fu's F_S-values (Table 3), suggesting that the lower ACO1I9 diversity in the Palaearctic did not result from selection or a recent bottleneck.

DISCUSSION

The geographical patterns in phenotypic and vocal variation in the pine grosbeaks (Adkisson, 1999) suggest that pine grosbeaks have multiple distinct lineages on different continents and within the Nearctic. Our ND2 data support intercontinental differentiation in the pine grosbeak. We found three geographically concordant clades. Two closely related (2.2% ML-corrected divergence) sister clades included northern Nearctic localities from Alaska to Newfoundland, and south-western Nearctic localities in mountains of Idaho, Colorado and California, respectively. In addition, the mtDNA gene tree of Topp & Winker (2008) showed that pine grosbeaks from the Queen Charlotte Islands were nearly reciprocally monophyletic, suggesting either a third Nearctic clade, albeit of limited distribution, or that the Queen Charlotte Islands population is related to the south-western Nearctic clade, with which they share sound characteristics (Adkisson, 1999). All Palaearctic sequences sampled from Scandinavia to the Pacific coast of Asia formed the third clade, which was distantly related (6.4% divergence) to the Nearctic clades. Therefore, similar to three-toed woodpeckers and winter wrens, Palaearctic and Nearctic pine grosbeaks have experienced a long-term isolation and have at least two divergent lineages in North America. However, in contrast to what is seen in these other two boreal forest Holarctic birds, the Nearctic mtDNA lineages of pine grosbeak are more closely related to each other, thereby obscuring the direction of intercontinental colonization.

Our ACO1I9 data also supported differentiation of Palaearctic pine grosbeaks from Nearctic birds. Palaearctic ACO1I9 alleles formed a distinct clade supported by a 67% bootstrap value. In contrast to what is observed in the mtDNA tree, the pattern of relationships among ACO1I9 clades was the same as in the three-toed woodpecker and winter wren [Nearctic (Nearctic, Palaearctic)], suggesting a Nearctic to Palaearctic direction of the intercontinental colonization. Lower π_n and a mean pairwise difference of 1.35 in the Palaearctic are also consistent with colonization of the Palaearctic from the Nearctic.

Differences in demographic history or selection pressures between the continents could provide alternative explanations for the position of the Palaearctic clade on the ACO1I9 tree and for differences in levels of genetic diversity between the Palaearctic and Nearctic. However, the geographical scale of our sampling and the biogeographical history of the Holarctic do not support these alternatives. For example, during the Last Glacial Maximum, the Nearctic was much more extensively glaciated than the Palaearctic, resulting in a greater contraction of boreal forests (Hewitt, 2004), which would favour a stronger Nearctic bottleneck. However, π_n is much greater overall in the Nearctic than in the Palaearctic. It is also difficult to explain why selection pressures resulting in intron hitchhiking are strong and similar across multiple biogeographical regions of the Palaearctic (e.g. western and eastern Palaearctic, western Beringia) but are much weaker in all biogeographical regions of the Nearctic, including eastern Beringia which shares its biogeographical history and is ecologically similar to western Beringia. Furthermore, Fu's F_S- and *R2*-tests conducted for each individual Palaearctic locality and for all Palaearctic ACO1I9 sequences combined provided no support for greater selection/hitchhiking on ACO1I9 sequences.

Although Nearctic ACO1I9 sequences formed two clades, in contrast to ND2 sequences, these clades were sympatric in both

northern boreal and south-western mountain forests. One possible explanation is the lack of lineage sorting as a result of insufficient time since isolation of the two geographical regions. Recently, Zink & Barrowclough (2008) argued that this is the most parsimonious inference in the case of 'disagreement' between mtDNA and nuclear sequence data when lineage sorting is complete for mtDNA sequences and nuclear sequences are not sorted. The comparison of levels of divergence between Palaearctic and Nearctic pine grosbeaks supports such an inference. The distance between the Palaearctic ACO1I9 clade and its Nearctic sister clade was 0.2%. This distance is 29 times shorter than the branch length between the Palaearctic and either of the Nearctic ND2 clades (6.4%). It is not surprising then that ACO1I9 sequences from the northern and south-west Nearctic were not sorted into clades, in contrast to the case for our ND2 sequences (Palumbi *et al.*, 2001).

An alternative explanation could invoke sex-specific differences in gene flow. This would require a nearly complete lack of female movement and virtually unrestricted movement of males between the northern and south-western Nearctic over extended periods. A great difference between sexes in gene flow levels for such a long time is required to produce 2.2% divergence between mtDNA clades and non-significant F_{ST}-values in a *Z*-specific locus. It is especially unlikely in this case because selection does not seem to have a strong effect on either locus. The MK test was unable to reject the neutrality of ND2 sequence evolution, and all ACO1I9 clades had non-significant Fu's F_S- and *R2*-values. Thus, the lack of time for geographical sorting of *Z*-specific alleles would seem the most parsimonious explanation.

The division of Nearctic pine grosbeaks into northern boreal and south-western mountain lineages, observed in the ND2 data, is consistent with the vocal differences between birds inhabiting these areas (Adkisson, 1999). As noted above, our ACO1I9 data did not support the differentiation of the two Nearctic groups, but it did not reject it (Zink & Barrowclough, 2008). The morphological and vocal differences (Adkisson, 1999) and our genetic data suggest that the taxonomic status of the three distinct lineages within the pine grosbeak needs a re-evaluation.

Not all Holarctic birds exhibit a phylogeographical structure that can be explained by decreased gene flow between continents. Most non-forest taxa are either not differentiated at all or have become isolated only recently, not allowing for complete lineage sorting (Table 1). Furthermore, in a few Holarctic taxa with a complex phylogeographic structure, for example the dunlin (*Calidris alpina*; Wenink *et al.*, 1996) and the barn swallow (*Hirundo rustica*; Zink *et al.*, 2006b), phylogeographical structure is better explained by non-Beringean biogeographical boundaries.

This study shows that the pine grosbeak has distinct mtDNA lineages in the Nearctic and the Palaearctic. This phylogeographical pattern agrees with that in the three-toed woodpecker (Zink *et al.*, 2002) and the winter wren (Drovetski *et al.*, 2004a), the only boreal forest birds with Holarctic distributions that have been adequately studied to date. The degree of mtDNA divergence in all three species suggests that their respective Nearctic and Palaearctic populations became isolated in the Late Pliocene or Early Pleistocene (depending on which rate of evolution is used to obtain this time estimate). These boreal forest populations apparently did not come into a secondary contact during any of the several latest glaciation cycles, despite the formation of the Bering land bridge. Long-term isolation of Nearctic and Palaearctic boreal forest populations is also suggested by the fact that only a few taxa are considered under current taxonomic designations to have conspecific populations on the two continents. In the majority of non-avian taxa with Holarctic boreal forest distributions, the Nearctic and Palaearctic populations are regarded as species pairs (Lafontaine & Wood, 1988). Forthcoming studies of the other putative boreal forest Holarctic bird species (goshawk, great grey owl, hawk owl, boreal owl, Bohemian waxwing, red crossbill, white-winged crossbill) will reveal if any are indeed single species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Museum and tissue numbers, sex, longitude (°E), latitude (°N), ND2 haplotypes, ACO1I9 alleles, and NCBI accession numbers for all species used in this study.

Appendix S2 Neighbour-joining tree for ND2 haplotypes of pine grosbeak (*Pinicola enucleator*) and all outgroups.

Appendix S3 Neighbour-joining tree for ACO1I9 alleles of pine grosbeak (*Pinicola enucleator*) and Eurasian bullfinch (*Pyrrhula pyrrhula*).

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BIOSKETCH

Sergei V. Drovetski has professional interests in molecular and morphological evolution, the genetics and ecology of speciation, functional morphology, behavioural ecology and biogeography. He has a special interest in Holarctic and high-latitude biogeography. S.V.D., R.M.Z. and I.V.F. have collaborated on studies of phylogenetics and phylogeography of Palaearctic and Holarctic birds for over a decade.

Author contributions: S.V.D. conceived the idea for this paper; S.V.D., R.M.Z., P.G.P.E. and I.V.F. collected samples and data; S.V.D. analysed the data; S.V.D., R.M.Z., P.G.P.E. and I.V.F. contributed to the writing.

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